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SO VET.MED. (76, NO.8, 1185-86, 1981).*

SO Vet.Med. (82, No. 6, 646-50, 1987) 2 Tab. 23 Ref. *

SO VETERINARY PARASITOLOGY, (MAY 1993) Vol. 47, No. 3-4, pp. 225-233.

SO Z PARASITENKD, (1983) 69 (1), 27-34.*

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SO Vet.Rec. (111, No. 18, 414-15, 1982) 2 Fig. 2 Tab. 12 Ref.*

SO J PROTOZOOL, (1987) 34 (4), 398-402.

SO AM J TROP MED HYG, (1987) 36 (3), 505-508*

Applied and Environmental Microbiology, (1995) Vol. 61, No. 2, pp.*

SO Journal of Parasitology, (Feb., 1998) Vol. 84, No. 1, pp. 8-15.

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SO FEMS Microbiology Letters, (1994) Vol. 118, No. 1-2, pp. 45-49.

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CRYPTOSPORIDIUM PARVUM DEVELOPMENT IN THE BS-C-1 CELL LINE

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ABSTRACT: *Cryptosporidium parvum* is a worldwide parasitic protozoon capable of causing life-threatening disease in immunocompromised patients. In vitro cultivation of *C. parvum* has been under investigation for development of a well-defined in vitro model for *C. parvum* infectivity assay. This is the first report of *C. parvum* completing its life cycle in BS-C-1, an African green monkey kidney cell line. Both sodium hypochlorite-stimulated oocysts and purified sporozoites were able to initiate infection that led to completion of the whole life cycle, although inoculating purified sporozoites was less efficient. Beside Giemsa staining and normal light microscopy, an indirect fluorescent antibody staining method was developed to facilitate the detection and interpretation of *C. parvum* life stages developed in vitro. A *C. parvum*-specific polymerase chain reaction was also applied to detect and confirm the presence of *C. parvum* life stages in liquid from oocyst-infected cell cultures. In addition to the 452-base-pair (bp) product that can be specifically amplified from *C. parvum* oocysts and sporozoites, a fragment near 280 bp was also obtained. Various applications of this in vitro culture system are envisioned.

Cryptosporidium parvum is an intracellular parasitic protozoon that infects most mammals, including humans. It causes a self-limited gastrointestinal illness in immunocompetent individuals and a life-threatening diarrheal disease in immunocompromised patients, especially those infected with human immunodeficiency virus (HIV), because no effective therapy for cryptosporidiosis is available (Fayer et al., 1990; Peterson, 1992). Although *C. parvum* was not recognized as a human pathogen until 1976 (Meisel et al., 1976; Nime et al., 1976), the significance of waterborne cryptosporidiosis has received worldwide recognition (D'Antonio et al., 1985; Hayes et al., 1989; Moore et al., 1994). In 1993, *C. parvum* caused the largest waterborne disease outbreak ever recorded in the United States (MacKenzie et al., 1994), and it has been gaining more and more attention since then.

To investigate the effects of environmental stresses and disinfection methods on survival of *C. parvum* oocyst, the infective and transmissive stage of the protozoa, as well as to evaluate the effectiveness of various water treatments in reducing the risk of contracting *C. parvum* from community and municipal water supplies, a simple and reliable infectivity assay is of great importance. Although in vitro excystation has been investigated extensively, and, as well as a fluorogenic dye staining-based viability assay, widely used for quantitative viability analysis (Fayer and Leek 1984; Woodmansee, 1987; Current, 1990; Campbell et al., 1992), the in vivo infectivity assay using a small animal model (Kwa et al., 1993; Fayer, 1995) is still the only reliable assay, because neither in vitro excystation nor fluorogenic dye-staining exactly reflects the in vivo infectivity of *C. parvum* oocysts. However, the in vivo infectivity assay is both time consuming and expensive, making it impractical and unaffordable for most routine studies.

The successful in vitro culture of *C. parvum* provided a suitable means for carrying out in vitro assay of pharmacological activities (McDonald et al., 1990); it also presented an alternative for *C. parvum* infectivity assay. Since it was first reported that *C. parvum* was able to complete its life cycle in a cultured cell line (Current and Haynes, 1984), many cell types have been found capable of supporting *C. parvum* growth and development to some extent (Eggleston et al., 1994; Upton, Tilley, and Brillhart, 1994; Yang et al., 1996). In this paper, we describe a simple system to initiate infection with *C. parvum* oocysts and complete the whole *C. parvum* life cycle in an African green monkey kidney cell line, which has not been reported to support *C. parvum* growth.

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MATERIALS AND METHODS

Purification and viability estimation of *C. parvum* oocysts

Cryptosporidium parvum oocysts were obtained from feces of infected calves on a dairy farm and purified using previously described methods (Current, 1990), with some modifications. The feces were homogenized in distilled water containing 0.01% Tween-20 (Tween-H_2O) and passed through a series of stainless sieves with the minimum porosity at 63 μm. After centrifugation at 1,000 *g* for 5 min, the sediment was suspended in Tween-H_2O, and 5 ml was applied over 20 ml Sheather's sugar solution (500 g sucrose and 6.5 g phenol in 320 ml distilled water, 1.20 g/ml) and centrifuged at 1,000 *g* for 10 min. The oocyst-containing layer was removed, pooled, washed once with Tween-H_2O, and once with Tris-buffer solution (50 mM Tris, 10 mM EDTA, pH 7.2), and resuspended in Tris-buffer solution. Oocyst suspension (0.5–1.0 ml) was overlaid to an ultracentrifuge tube containing a CsCl gradient that was made by sequentially adding 1.5 ml CsCl solutions in the following gradient order: 1.40 g/ml, 1.10 g/ml, and 1.05 g/ml (all of these CsCl solutions were prepared from 1.80 g/ml CsCl stock solution by diluting with Tris-buffer solution). After centrifugation at 16,000 *g* for 1 hr at 10 C in an L8–80M ultracentrifuge (Beckman Instruments Inc., Palo Alto, California) using a Ti-40 rotor, the oocyst-containing band (between 1.10 g/ml layer and 1.05 g/ml layer) was carefully removed and washed twice in Tris-buffer solution to remove the residual CsCl solution, followed by washing with Tween-H_2O at 1,000 *g* for 10 min. Oocysts were resuspended in 1× Hank's balanced saline solution (HBSS, Sigma Chemical Co., St. Louis, Missouri) containing 25 U/ml penicillin and 25 μg/ml streptomycin, enumerated on a bright-line hemacytometer (Hausser Scientific, Horsham, Pennsylvania), and stored at 4–10 C.

A standard in vitro excystation procedure (Campbell et al., 1992) was performed to monitor the viability of the oocysts to be used for cell infection. Briefly, 100 μl oocyst suspension (~10^6/ml in 1× HBSS) was mixed with 200 μl of freshly prepared 1% bovine bile (Sigma) and 50 μl of 0.44% $NaHCO_3$ solution, incubated at 37 C for at least 4 hr, and examined under a normal light microscope. A total of 100 oocysts was counted, and the percentage excystation efficiency (viability) was calculated as: excystation efficiency (%) = number of empty oocysts + number of partially excysted oocysts − baseline (number of empty oocysts among 100 oocysts before excystation).

Production and specificity test of anti-*C. parvum* sera

Anti-*C. parvum* serum was raised by immunizing a 2.5–3.0-kg female New Zealand white rabbit with 1.0 × 10^7 purified oocysts. After over-

night treatment with 10% formalin solution and extensive washing to remove the formalin residue, the oocysts were resuspended in 3.0 ml phosphate-buffered saline (PBS, pH 7.2). For the first immunization, 1.0 ml antigen preparation with complete Freund's adjuvant was injected into the rabbit subcutaneously at multiple sites. Control serum was obtained before the injection. On days 12 and 25 after the first injection, an additional 1.0 ml of the antigen mixture was injected with incomplete Freund's adjuvant. Starting from day 10 after the final injection, the rabbit was bled at 1-wk intervals, the sera were titrated by a conventional slide agglutination test, and the rabbit was anesthetized and exsanguinated after the serum titer achieved a satisfactory level (1:400 working dilution). In the slide agglutination test, 100 μl of purified oocyst suspension was placed on an agglutination slide (American Scientific Products, McGaw Park, Illinois); 100 μl of rabbit serum dilutions in PBS (pH 7.2) was added to the suspensions and mixed with a wooden applicator stick. The slides were rotated by hand for 45 sec, after which they were incubated at room temperature for up to 2 hr and the suspension was observed for agglutination. A negative control using preimmunization rabbit serum was performed with each run; the agglutination was graded on a 1–4 scale and confirmed by examination under a light microscope equipped with long working distance objectives.

An indirect fluorescent antibody staining (IFAS) technique was used to determine the reaction specificity of immune serum. A drop (~50 μl) of either purified *C. parvum* oocyst suspension, a mixture of *C. parvum* oocysts and sporozoites (obtained from in vitro excystation), *Cryptosporidium muris* oocysts (purified from a fecal sample), *Giardia duodenalis* (ATCC 53098, American Type Culture Collection, Rockville, Maryland), or BS-C-1 cells was placed on a fluorescent antibody slide (Becton Dickinson, Franklin Lakes, New Jersey) coated with 0.1% poly-L-lysine (Sigma), air dried, and fixed in acetone for 5 min at room temperature. Then, 50 μl of the working dilution of immunized rabbit serum was applied to the spots and incubated for 30 min at 37 C in a humidified chamber. After washing with PBS, 50 μl of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Sigma) was added, and the slides were incubated for another 30 min at 37 C in the humidified chamber. The slides were washed again with PBS and examined with a standard fluorescence microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with epi-illumination, 100 W halogen bulb, band pass 450–490 nm (blue) exciter filter, 510 nm chromatic beam splitter, and long pass 520 nm barrier filter. The staining reactions were recorded as "positive" or "negative."

Preparation of BS-C-1 cells

BS-C-1 cell (ATCC CCL26, ATCC), a continuous cell line that originated from African green monkey (*Cercopithecus aethiops*) kidney cells, was obtained and previously used for virus propagation and titration (Deng and Cliver, 1992). Cells were grown in 75-cm² tissue culture flasks (Corning Glass Works, Corning, New York) at 37 C in a 5% CO_2–95% air humidified incubator in Eagle's minimum essential medium (Sigma) supplemented with 2.0 mM L-glutamine (GIBCO BRL Life Tech., Gaithersburg, Maryland) and 10% fetal bovine serum (FBS, Sigma). The same medium with 5% FBS was used for maintenance. For *C. parvum* infection, BS-C-1 cells were harvested with 1× trypsin-EDTA (GIBCO BRL) for 2 min at 37 C, suspended in growth medium, counted, distributed in 24-well, flat-bottom plastic tissue culture plates (Corning) with a 12-mm circular coverglass (Fisher Scientific, Fair Lawn, New Jersey) placed in the bottom of each well. Each cover slip received 1.0–1.5 × 10^5 cells, so that ~90% confluency was achieved after 24 hr incubation. Immediately before inoculation of *C. parvum*, growth medium was aspirated.

Infection of BS-C-1 monolayer cells with *C. parvum* oocysts

The method described by Upton, Tilley et al. (1994) was used with some modifications. Before use, purified oocysts were washed twice with distilled water and incubated with freshly prepared 10% (v/v, full strength is 5.25% sodium hypochlorite) Clorox® bleach (The Clorox Company, Oakland, California) for 10 min on ice. After being washed twice with ice-cold distilled water and once with ice-cold BS-C-1 cell maintenance medium, oocysts were resuspended in 37 C maintenance medium at 2.0 × 10^5/ml; 2.0 ml was inoculated to each well. Several wells receiving 2.0 ml maintenance medium containing no oocysts served as negative controls. The plates were incubated at 37 C for 3 hr in a candle jar (desiccator) to allow oocysts to excyst and sporozoites to adsorb onto cells, after which the unexcysted oocysts, empty oocyst walls, and noninfectious sporozoites were removed by 3 washings with prewarmed (at 37 C) PBS. Fresh maintenance medium (2.0 ml) was added, and the plates were put back into the candle jar and incubated at 37 C. The maintenance medium was changed after 72 hr.

Infection of BS-C-1 monolayer cells with *C. parvum* sporozoites

To compare the growth and development of *C. parvum* in BSC-1 using sporozoites as inoculum, bleach-stimulated oocysts were incubated in prewarmed (at 37 C) maintenance medium for 30 min, sporozoite release was confirmed by microscopical examination, and the sporozoites were separated from intact oocysts and empty oocyst walls by gently passing the excystation mixture through a sterile 2.0-μm polycarbonate filter (Millipore Corporation, Bedford, Massachusetts). The sporozoites were washed once at 1,000 *g* for 10 min and resuspended in maintenance medium at 4 × 10^5/ml, 2.0 ml was inoculated to each well, and the cells were incubated in the candle jar at 37 C for 3 hr. The extracellular sporozoites were then removed by 3 washings, and the cells were reincubated in the candle jar at 37 C as described above.

Observation of BS-C-1 cells infected with *C. parvum* oocysts/sporozoites

To determine the extent of *C. parvum* colonization and development, coverslips containing infected cells were removed at 5, 12, 24, 48, 72, 96, and 120 hr postinfection (PI), respectively; gently washed with prewarmed PBS; placed in absolute methanol at 4 C for 10 min for fixation; stained with modified Giemsa stain (Sigma) at room temperature for 45 min; washed 3 times in distilled water; air dried; wet-mounted; and viewed under a bright-field microscope under oil immersion (×1,250 magnification). Coverslips containing uninfected BS-C-1 cells were processed in the same way as negative controls.

An IFAS procedure, similar to that used in the antiserum specificity test, was applied to confirm the presence of different intracellular *C. parvum* life stages. At selected intervals, coverslips were removed, washed gently with prewarmed PBS, fixed in acetone at −20 C for 20 min, air-dried, and incubated with 1.0 ml of 1:100 dilutions of rabbit anti-*C. parvum* serum at 37 C for 30 min. After washing 3 times with PBS, 1.0 ml of 1:160 dilution of FITC-labeled goat anti-rabbit IgG was added; and the coverslips were incubated at 37 C for another 30 min, washed 3 times with PBS, and viewed under the fluorescent microscope at ×1,250 magnification. Coverslips containing uninfected BS-C-1 cells were processed in the same way as negative controls.

Examination of oocyst-infected cell culture liquids

To detect the possible presence of *C. parvum* life stages released into cell culture fluids, media from wells infected with oocysts were pooled into a centrifuge tube after 72 hr PI and the sediments from the media were washed twice, resuspended in PBS, stained with rabbit anti-*C. parvum* serum and FITC-labeled goat anti-rabbit IgG, and viewed under the fluorescent microscope. Alternatively, the collected media were directly viewed under a bright-field light microscope.

PCR to detect *C. parvum* life stages from oocyst-infected cell culture liquid

Primers and PCR parameters: Based on the sequence reported by Laxer et al. (1991), two 26-mer primers (upstream primer: 5′-CCGAGTTTGATCCAAAAAGTTACGAA-3′ and downstream primer: 5′-TAGCTCCTCATATGCCTTATTGAGTA-3′) were used to amplify a 452-base-pair (bp) fragment within an undefined *C. parvum* DNA region. The PCR mixture (50 μl), containing 1× PCR buffer (Perkin-Elmer Cetus Corp., Norwalk, Connecticut), 200 μM of each of the 4 deoxynucleoside triphosphates (dNTPs, Perkin-Elmer), 1.0 μM of both primers (GeneMed Biotech., San Francisco, California), 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer), and DNA template was overlaid with 25 μl mineral oil (Sigma) to prevent evaporation. A negative control without DNA template was included in each batch to monitor possible cross contamination. PCR was performed on a Progene 120 Thermal Cycler (Techne Inc., Princeton, New Jersey). The reaction mixture was initially denatured at 94 C for 3 min, then subjected to 35 cycles of denaturation at 94 C for 1 min, annealing at 50 C for 1 min,

and extension at 72 C for 1 min, with an additional 7-min extension at 72 C.

Determination of PCR specificity: The specificity of the primers and PCR reaction was tested by attempting to amplify target sequences from the following heterologous organisms: *C. muris, G. duodenalis, Escherichia coli* O157:H7 (ATCC 43897), and BS-C-1 cells. DNAs from *C. muris, G. duodenalis,* and BS-C-1 were isolated by a modified proteinase K method as described below, whereas *E. coli* DNA was released by heating ~1 × 10^6 cells (in 25 μl distilled water) at 95 C for 10 min and cooling to 4 C for 5 min. The extracted DNAs were then used in PCR as templates.

DNA extraction for PCR amplification: C. parvum oocyst DNA was extracted by a modified proteinase K method. Briefly, ~1 × 10^6 oocysts were pelleted, washed, and resuspended in 500 μl 1× Tris-EDTA buffer (pH 8.0) containing 100 μg/ml proteinase K (Promega Corp., Madison, Wisconsin) and 0.8% *N*-lauroyl sarcosine (Sigma). After digestion at 58–60 C for 4 hr, the supernatant was mixed with equal volume of phenol : chloroform : isoamyl alcohol (25:24:1, Fisher Scientific), and the aqueous phase was transferred following a 5-min centrifugation at 10,000 *g*. DNA was precipitated with equal volume of ice-cold isopropanol in the presence of 200 μg/ml D-glycogen (Fisher Scientific), washed with 70% alcohol, and resuspended in 100 μl distilled water.

To extract DNA from pure sporozoites, ~1 × 10^6 *C. parvum* oocysts were incubated in excystation solution containing bovine bile and $NaHCO_3$ at 37 C for 2 hr, and sporozoites were purified as described earlier. After washing the sporozoite suspension twice and resuspending in 100 μl distilled water, DNA was extracted by heating to 95 C for 10 min and cooling to 4 C for 5 min. Following centrifugation at 10,000 *g* for 10 min, the supernatant was transferred and used in PCR.

DNA extraction from oocyst-infected cell culture liquids was performed similarly; e.g., cell culture liquids were collected, washed twice, resuspended in 100 μl distilled water, and incubated at 95 C for 10 min and then at 4 C for 5 min. Liquids from uninfected cell cultures were also collected and processed in the same way, as an additional negative control.

Analysis of *C. parvum* PCR products

Following PCR, portions (10–30 μl) of the reaction mixtures were separated on 2.0% agarose in 1× Tris–acetate–EDTA (TAE, Sigma) buffer stained with 0.5 μg/ml ethidium bromide, and viewed with a UV transilluminator. A 1-kb DNA ladder (GIBCO BRL) was included as a DNA size marker, to determine the sizes of amplification products.

To characterize the PCR products, a nonradioactive oligonucleotide hybridization, using an internal probe sequence (5′-GAA/TTA/ACC/TAT/AGG/AAC/CT-3′) within the template sequence, was performed. The Southern hybridization procedure described previously (Bugawan et al., 1988) was followed with modifications: PCR products were separated on a 2.0% agarose gel with a biotinylated ϕX174/Hinf I fragment (GIBCO BRL) as a DNA size marker, transferred overnight to Zeta-Probe® Blotting membrane (Bio-Rad Lab., Hercules, California) by capillary transfer in 0.4 N NaOH, and fixed in an FB-UVXL-1000 microprocessor-controlled UV crosslinker (Fisher Scientific) at 120 mJ/cm² (optimal setting). The membrane was prehybridized in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7), 5× Denhardt's solution (Sigma), 0.5% sodium dodecyl sulfate (SDS, Sigma) for 30 min at 50 C. Internal probe (10 pmol, 5′-biotin-labeled; GeneMed) was added, and hybridization was performed at the same temperature for 1 hr. Subsequently, the membrane was washed at room temperature for 10 min in 2× SSPE, 0.1% SDS; at 50 C for 20 min in 5× SSPE, 0.5% SDS; and at room temperature for 10 min in 2× SSPE. The membrane was then incubated with streptoavidin-peroxidase (POD) conjugate (Boehringer Mannheim, GmbH, Germany) at 0.5 U/ml (in 2× SSPE) at room temperature for 20 min. After 2 5-min washes in 100 mM NaCl, 1 M urea, 0.5% Triton X-100, and 1% dextran sulfate (Sigma), followed by 2 5-min washes in 100 mM sodium citrate (pH 5.0), color was developed by incubating the membrane in 100 mM sodium citrate (pH 5.0) containing 0.1 mg/ml 3, 3′, 5, 5′-tetramethylbenzidine (TMB, Sigma) and 0.03% H_2O_2 (added immediately before use) at room temperature for 10 min. The color reaction was terminated by washing the membrane in distilled water for 2 min.

As an alternative identification method, the amplification products were also subjected to restriction enzyme analysis based on the sequence data that an unique StyI site (C↓CAAGG) is also contained in the template sequence. Samples (10 μl) from amplification reactions were added into the digestion mixture containing 1.0 μl StyI enzyme (New England Bio-Labs, Inc., Beverly, Maryland), 2.0 μl 10× NEBuffer 3 (New England Bio-Labs), 0.2 μl 100× bovine serum albumin (BSA, New England Bio-Labs), and 6.8 μl distilled water; incubated at 37 C for 1 hr; and examined by gel electrophoresis and ethidium bromide staining.

RESULTS

Cryptosporidium parvum oocysts and anti-*C. parvum* serum

Oocysts of high purity and viability were obtained by Sheather's sugar flotation, followed by CsCl gradient centrifugation. Successful excystation was demonstrated by scanning electron microscopy (SEM) in an earlier study (Deng et al., 1997). Because there was no significant decrease in viability during an 8-wk storage in 1× HBSS at refrigeration temperature, oocysts used throughout the study had an 88–90% excystation efficiency. The rabbit antiserum was found to be *C. parvum* specific, as there was no cross reaction with *C. muris, G. duodenalis,* or BS-C-1 cells. However, it did react with both *C. parvum* oocysts and sporozoites.

Observation of host cells infected by *C. parvum* oocysts/sporozoites

By Giemsa staining, successful *C. parvum* development was observed in BS-C-1 cells inoculated with *C. parvum* oocysts (Fig. 1). At 5 hr PI, intermediate *C. parvum* life stages, mainly trophozoites and a few meront early stages, were found in an intracellular location, usually close to the nucleus of the host cell. As the incubation continued, more and later life stages were also observed. At 12 hr PI, more meronts (type I and type II), microgamonts, and macrogamonts were found. Progeny oocysts appeared by 24 hr PI. Multiple infections were not unusual, with some cells having more than 1 form of the parasite. However, by Giemsa staining, we were unable to distinguish type I meronts from type II meronts, or microgamonts from macrogamonts.

IFAS of infected cells also confirmed the presence of various *C. parvum* life stages inside the host cells (Fig. 2). Compared with Giemsa staining, usually more *C. parvum* life stages were observed in a field, and they were more distinguishable due to the specific antigen–antibody reaction and the dark background. But unlike in Giemsa staining, trophozoites, meronts, macrogamonts, and microgamonts were not stained as an integrated structure; rather, the individual trophozoites, merozoites, and gametes inside were stained after the host cells' surface membranes were destroyed by acetone fixation.

In an attempt to investigate the relationship between PI time and *C. parvum* development, we compared the total numbers of all *C. parvum* life stages at different intervals by randomly counting 25 oil-immersion fields per coverslip. By both Giemsa staining and IFAS, the total number increased as the incubation continued. At 48 hr PI, there was the highest number of all life stages, whereas the highest number of progeny oocysts was obtained at 72 hr. Prolonged incubation usually gave rise to higher number of microgamonts or macrogamonts, and subsequently higher number of progeny oocysts. However, after 72 hr PI, the total number began decreasing, partly because the host cells started sloughing and some *C. parvum* life stages

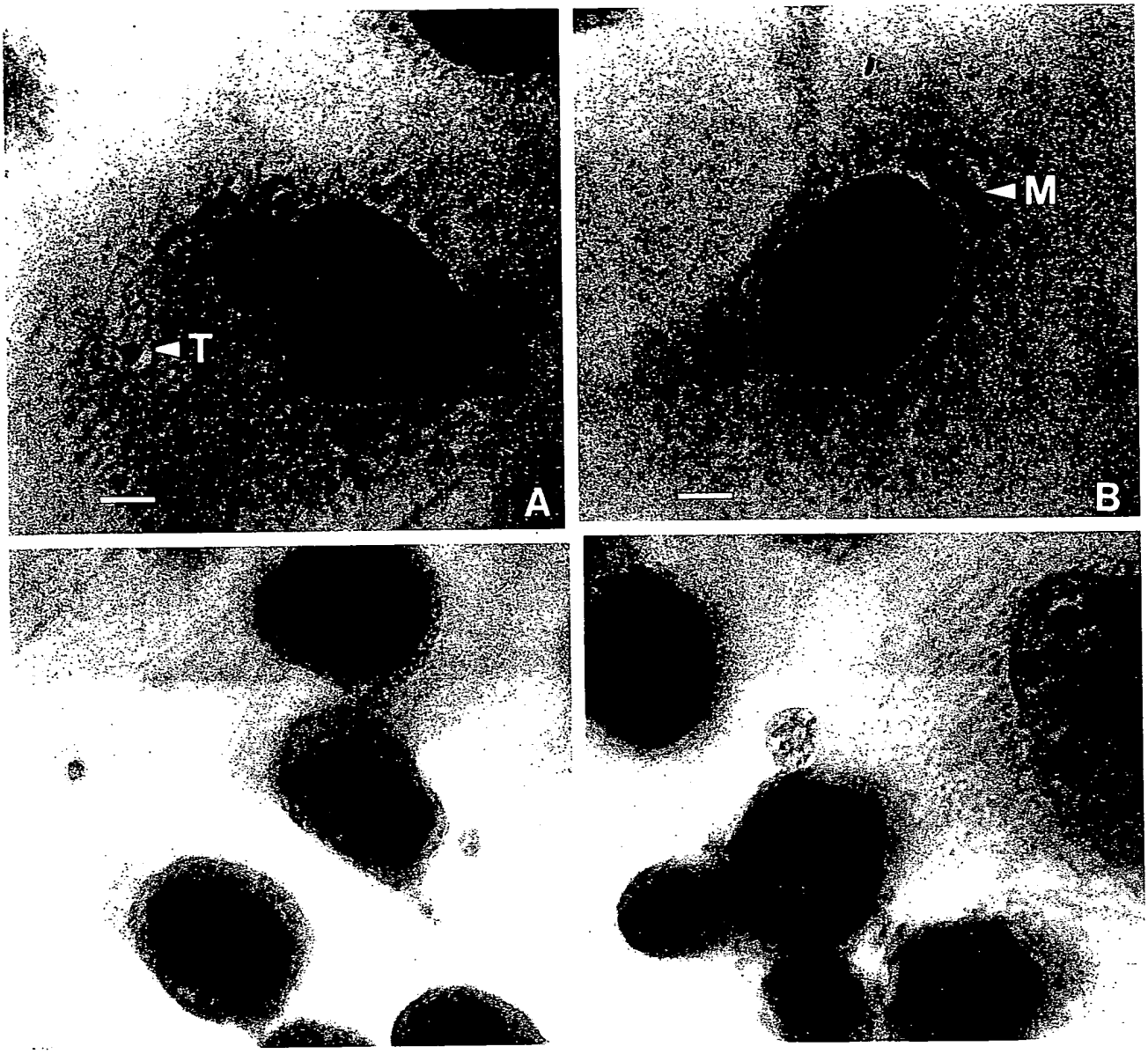

FIGURE 1. Differentiated *C. parvum* life stages seen by Giemsa staining of BS-C-1 cells infected with *C. parvum* oocysts. (A) Trophozoite (T); (B) type I or type II meront (M); (C) microgamont or macrogamont (MG); (D) progeny oocyst (PO). Bars = 5 μm.

might be released into the cell culture liquids. Therefore, the total number of counted *C. parvum* life stages dropped dramatically at 96 hr and 120 hr PI.

Cryptosporidium parvum development and growth in BS-C-1 cells infected with purified sporozoites was similar; however, lower numbers of intermediate life stages or progeny oocysts were counted by Giemsa staining or IFAS.

Examination of oocyst-infected cell culture liquids

The hypothesis that prolonged incubation will cause release of some *C. parvum* life stages into the cell culture liquid was confirmed. IFAS revealed the presence of progeny oocysts, individual merozoites, trophozoites, gametes (due to the similarity in size and morphology, they were difficult to be distinguished from each other), clusters of merozoites, and surprisingly, some sporozoites, in the cell culture liquids. These life stages, as well as meronts, were also detected by direct light microscopy. An unusual observation under the light microscope was that >80% of the progeny oocysts were empty or partially empty; occasionally, some oocysts were in the process of excysting with a sporozoite projecting from a suture.

PCR to detect presence of *C. parvum* life stages in infected cell culture liquids

The PCR amplification was found to be highly specific for *C. parvum.* When heterologous template DNA from *C. muris, G. lamblia, E. coli,* or BS-C-1 cells was used, no amplification product was detected, whereas an otherwise identical reaction mixture containing *C. parvum* template DNA gave the specific

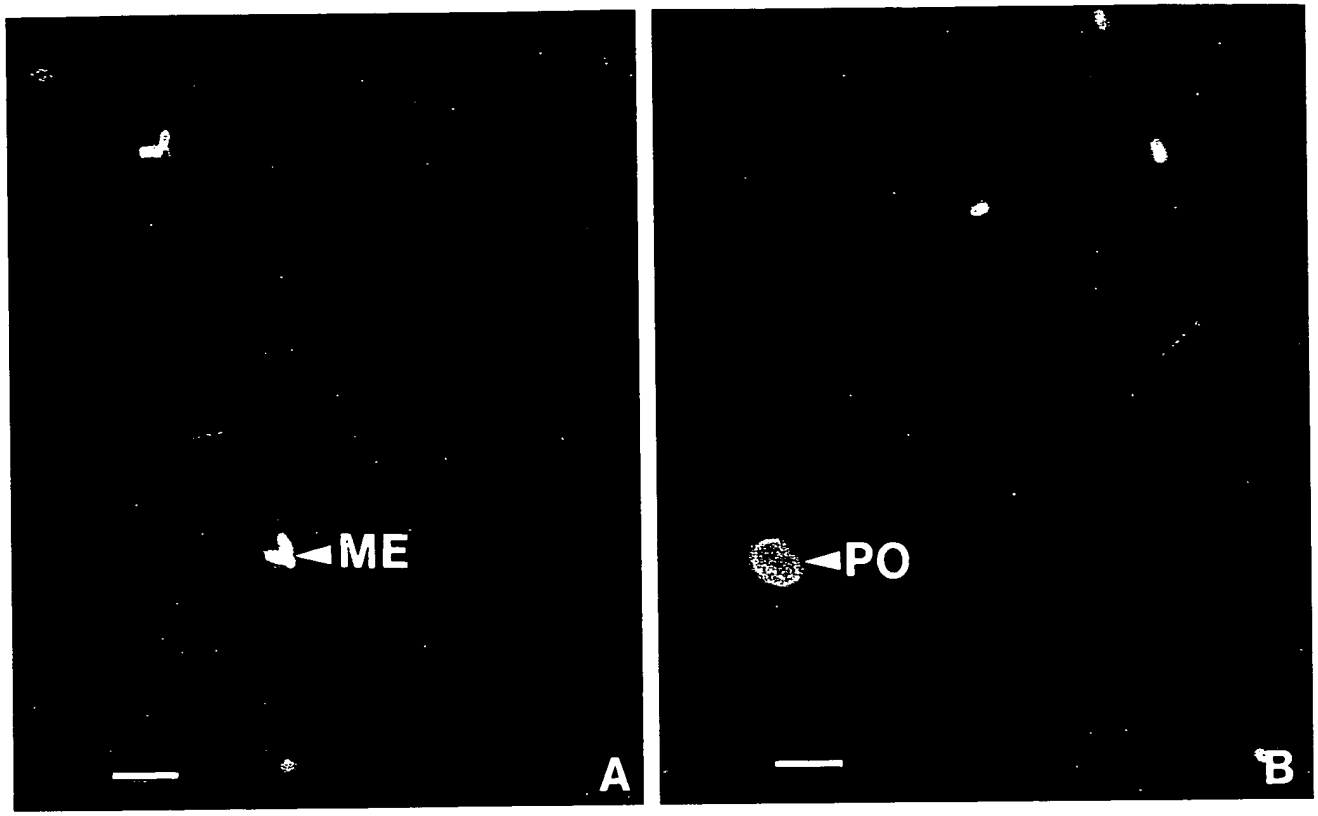

FIGURE 2. Differentiated *C. parvum* life stages seen by indirect fluorescent antibody staining (IFAS) of BS-C-1 cells infected with *C. parvum* oocysts. (A) Merozoites (ME); (B) progeny oocyst (PO). Bars = 5 μm.

452-bp product. Thus, a signal from a PCR reaction is the result of specific primer annealing and amplification.

When DNA extracted from oocysts or sporozoites was used as template, the 452-bp fragment was consistently amplified (Fig. 3, lanes 1 and 2). However, when DNA extracted from oocyst-infected cell culture liquids was used, the main PCR product was, by comparison with the DNA size marker, about 280 bp (Fig. 3, lane 3). Because the negative control using uninfected cell culture liquids did not give any signal (Fig. 3, lane 4), this band was attributed to specific amplification from parasite DNA templates present in cell culture liquids.

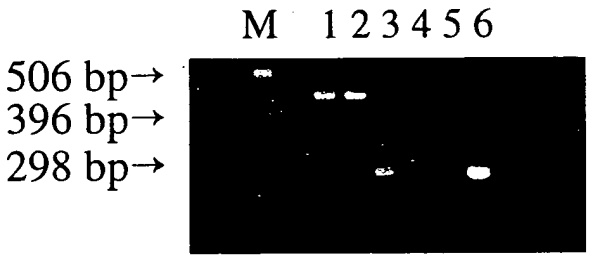

FIGURE 3. PCR to detect *C. parvum* life stages from oocyst-infected cell culture liquid. M, DNA size marker (1 kb DNA ladder); lane 1, DNA extracted from oocysts; lane 2, DNA extracted from purified sporozoites; lane 3, DNA extracted from infected cell culture liquids, notice that there is also a faint 452-bp band; lane 4, DNA extracted from uninfected cell culture liquids; lane 5, PCR negative control, no DNA template was contained in PCR mixture; lane 6, core sample PCR, in which purified ~280-bp PCR product was used as template in a second round PCR.

A core sample PCR procedure was applied to further test the specificity in amplifying 280-bp fragment. Briefly, the gel slice containing the band was cut, melted in 1× Tris-EDTA buffer (pH 8.0) at 65 C for 10 min, extracted by Tris-buffered phenol (Fisher Scientific), precipitated with isopropanol and glycogen, washed with 70% ethanol, and suspended in 100 μl distilled water; 0.5 μl was used as template in a second round PCR. As shown in lane 6 of Figure 3, a specific band was obtained at the same position, indicating the specificity of PCR amplification.

It is also noticeable that beside the 280-bp product, there was a faint 452-bp band when oocyst-infected cell culture liquids were used for PCR (Fig. 3, lane 3). This might be explained by the fact that both oocysts and sporozoites were also present in the cell culture liquid but at a lower level compared to other life stages. Coincidently, when oocyst-infected cell culture liquids were collected at 24 or 48 hr PI and used in PCR, the 452-bp band was stronger than the 280-bp band (data not shown).

Identification of PCR products

The results from nonradioactive oligonucleotide hybridization are shown in Figure 4. As expected, a signal was obtained from the 452-bp product (lane 1), demonstrating the effectiveness of the procedure and confirming the specificity of 452-bp amplification product. But there was no signal from the 280-bp product (lane 2), indicating the absence of the short oligonucleotide probe sequence.

Because the StyI site is located between bp 570 and 575,

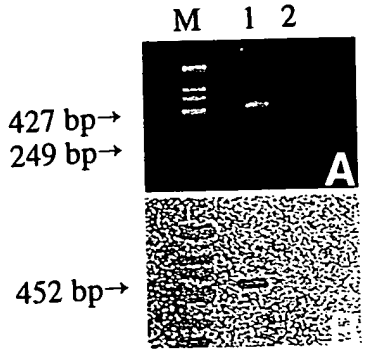

FIGURE 4. Identification of *C. parvum* PCR products: nonradioactive oligonucleotide hybridization. (A) DNA separation on 2.0% agarose gel stained with ethidium bromide; (B) Southern hybridization with biotin-labeled internal probe. M, DNA size marker (biotinylated ϕX174/Hinf I fragment); lane 1, 452-bp PCR product; lane 2, ~280-bp PCR product.

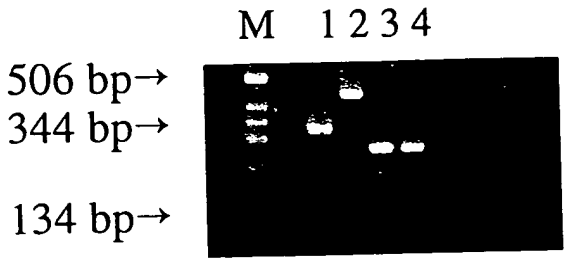

FIGURE 5. Identification of *C. parvum* PCR products: restriction endonuclease digestion. M, DNA size marker (1 kb DNA ladder); lane 1, 452-bp product after digestion with StyI, notice that the digestion was complete and a 325-bp and a 127-bp band were produced; lane 2, 452-bp product not subjected to StyI digestion; lane 3, ~280-bp product after digestion with StyI; lane 4, ~280-bp product not subjected to StyI digestion.

cleavage of the 452-bp (bp 444–895) PCR product by StyI will produce a 127-bp fragment (bp 444–570) and a 325-bp fragment (bp 571–895), as was confirmed in Figure 5 (lane 1). However, the 280-bp product cannot be digested by StyI (lane 3), suggesting that the fragment does not contain the restriction site for StyI.

DISCUSSION

Although in vitro cultivation of *C. parvum* has been researched for years, free sporozoites were used in most systems to infect cells, which raised some uncertainties regarding the reproducibility of the system and the degree of infection, because the viability of sporozoites outside the oocyst is not assured during the sporozoite purification process. In our system, BS-C-1 cell monolayers were infected with chemically stimulated *C. parvum* oocysts; a higher rate of infection was obtained compared with using purified sporozoites as inoculum, because the newly excysted sporozoites have immediate access to host cells and, as a result, spend minimal time exposed to the extracellular environment. Additionally, the system was highly reproducible due to the simplicity of the procedure. The excystation ratios of sodium hypochlorite-treated oocysts were found to be nearly identical to that of the standard excystation procedure using bovine bile salt and $NaHCO_3$, so the in vitro infection process was believed to mimic the in vivo process.

When purified sporozoites were used as inoculum, the presence of progeny oocysts inside host cells was proof that the whole *C. parvum* life cycle was completed in vitro. When oocysts were used in infecting host cells, the intracellular oocysts detected by Giemsa staining or IFAS and the presence of excysting oocysts and emerging sporozoites in infected cell culture liquids were direct evidences of successful *C. parvum* development in BS-C-1 cells.

In a preliminary experiment, we examined the oocyst-infected cells immediately after removal of noninfectious sporozoites and unexcysted oocysts: no residual oocysts were detected by either Giemsa staining or IFAS. Therefore, the oocysts detected at 24 hr PI or later were progeny oocysts, because they were located intracellularly (Figs. 1d, 2b). On the other hand, residual oocysts would persist extracelluarly if not removed during the multiwashing steps in IFAS. The observation of sporozoites and excysting oocysts in the cell culture liquids after 72 hr of incubation was also indicative. Sporozoites are very fragile, as was evidenced by previous reports of rapid lysis of sporozoites over the duration of in vitro excystation (Woodmansee, 1987; Campbell et al., 1992). The sporozoites present in the cell culture fluids were not residuals as the residuals would be unable to persist such a long time. Instead, they must result from oocyst excystation in the cell culture medium. Similarly, the excysting oocysts were not residuals either because excystation of sodium hypochlorite-stimulated oocysts would not last or take place after 72 hr incubation. Therefore, the only reasonable explanation was that these oocysts were progeny oocysts capable of excysting in cell culture medium without stimulation (evidently so-called thin-walled oocysts).

In most previous studies (Eggleston et al., 1994; McDonald et al., 1990; Upton, Tilley et al., 1994; Yang et al., 1996), the observation and interpretation of different stages in the parasite life cycle developed in vitro have relied on Nomarski-interference contrast microscopy or normal optical microscopy. These microscopic observations are based on the morphology and size of *C. parvum* life stages and usually are hard to interpret because these are undistinguishable from other intracytoplasmic vesicles or intracellular particles. In an attempt to compare our interpretations obtained from Giemsa-stained infected cells with the in vitro findings obtained by other authors, we found that in many cases, the observed forms were difficult to ascribe to a specific stage because they were either undifferentiated or they were in intermediate stages of differentiation. Therefore, we used the specific IFAS technique to detect and confirm the presence of some specific life stages. In our experience, IFAS was more reliable than Giemsa staining or phase-contrast light microscopy, because the FITC-labeled *C. parvum* life stages were easily distinguished from other intracellular particles due to the high specificity of the serum.

The anti-*C. parvum* serum used in our study reacted not only with oocysts and sporozoites but also with unwrapped trophozoites, merozoites, and gametes, confirming the high degree of antigen conservation among *C. parvum* life stages. In the future, if antibodies against specific life stages are available, the interpretation of different *C. parvum* development stages will become much easier and less arbitrary than by conventional microscopic observation.

In the present study, *C. parvum*-specific PCR was also used to detect and confirm the presence of *C. parvum* life stages in

infected cell culture liquids. The specificity of PCR was provided by the sequence-specific primers and confirmed by its inability to amplify DNA from organisms other than *C. parvum*. The consistent presence of the 452-bp amplicon confirmed the presence of *C. parvum* oocysts and sporozoites in the infected cell culture medium at 72 hr PI, which strongly confirmed the accomplishment of whole *C. parvum* life cycle in vitro. The simultaneous amplification of another fragment around 280 bp was also remarkable. Restriction enzyme analysis and oligonucleotide hybridization are frequently used for the identification and characterization of PCR products and, in our study, the results showed that the 280-bp fragment was quite different from the 452-bp fragment. Because we did not get any amplification product from uninfected cells' culture medium, the fragment was believed to be from the parasite, unlikely from BS-C-1 cells or any contaminating bacterium. Because this band cannot be obtained from oocysts or sporozoites, the template fragment is probably present in some other *C. parvum* life stages, such as meronts, microgamonts, or macrogamonts. Although more studies are required before any other conclusions can be made on such issues as the relationship between the 280-bp and the 452-bp fragments, the presence of the 280-bp fragment and specific *C. parvum* life stage development, and the possibility of DNA modification during *C. parvum* development, the combination of in vitro cultivation with PCR provides another possibility for further research on *C. parvum* life stage development or the host–parasite relationship at the molecular level.

The simplicity and effectiveness of the described system make it possible to develop a cell culture and enzyme-linked immunosorbent assay (ELISA)-based quantitative assay. After infecting the cells grown in a 96-well microplate with stimulated oocysts, *C. parvum* development can be interrupted at 48 hr PI (because the highest numbers of total *C. parvum* life stages were observed at that time), the cells can be stained with polyclonal antibodies against *C. parvum*, then stained with a second antibody conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (AP), followed by specific color reactions using appropriate substrates. Subsequent optical density (OD) measurement would give a quantitative assessment of the in vitro development of *C. parvum*. This assay will afford an in vitro test of infectivity to investigate effects of disinfection methods on oocyst viability by comparing the results from treated oocysts with those from untreated oocysts. By far, most in vivo infectivity determinations of *C. parvum* have been done in suckling mice (Lorenzo-Lorenzo et al., 1993; Fayer, 1995; Harp et al., 1996), which is difficult, costly, and requires up to 2 wk to complete. Although the readout time could be reduced to 6 days by using immunofluorescent flow cytometry (Venczel et al., 1997), the assay will become more expensive considering the limited availability of flow cytometry equipment and, like all other suckling mouse assays, it is not quantitative because a sample is simply categorized as "positive" or "negative." Moreover, the reported 50% infectious dose (ID_{50}) in a suckling mouse assay is from 60 to 1,000 oocysts (Ernest et al., 1986; Riggs and Perryman 1987; Korich et al., 1990; Finch et al., 1993), whereas humans can be infected with smaller numbers than this (Dupont et al., 1995). Although quantitative determinations of the sensitivity of the cell culture system have not yet been attempted, the practical infectious dose appears to be fewer than 10 oocysts, due to the high sensitivity of the ELISA, and the readout time can be 48 hr or less. Obviously, this will be more effective and less time consuming than in vivo assays. The same system could also be used in selecting drugs with anti-cryptosporidiosis effects, as has been described (Woods et al., 1995).

The in vitro cell culture system can also be used in detecting infective *C. parvum* oocysts from environmental samples. We have reported a technique to specifically detect viable *C. parvum* oocysts (using excystation as the viability criterion) by immunomagnetic capture PCR (IC-PCR) in which *C. parvum* oocysts were concentrated from an environmental sample by immunomagnetic separation (IMS) followed by in vitro excystation and PCR to amplify the 452-bp fragment (Deng et al., 1997). The IMS procedure can be used in association with the cell culture ELISA assay. The *C. parvum* oocysts would be captured from a sample by IMS and an acid/base treatment could be applied to detach oocysts from the magnetic particles. After stimulation by sodium hypochlorite, the oocyst suspension could be added to monolayer BS-C-1 cells followed by the standard cell culture ELISA to detect the presence of *C. parvum* life stages. This detection system will be more sensitive than conventional methods such as acid-fast staining (AFS) and immunofluorescent assay (IFA) because, unlike in other detection procedures, a single viable oocyst will give rise to four sporozoites and, by invading cells and subsequent multiplication, more life stages will be present when ELISA is performed. More significantly, unlike the conventional AFS or IFA, as well as most PCR-based methods, this system will effectively differentiate infective oocysts from noninfective ones. Hence, the IMS cell culture ELISA assay will not only sensitively detect and monitor possible contamination of *C. parvum* oocysts in the environment, such as surface water, but also give a more accurate assessment of the potential public health hazard of a *C. parvum*-contaminated water source, as the ability of *C. parvum* oocyst to complete the life cycle in vitro more precisely reflects its in vivo infectivity.

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